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Sporopollenin Content of the Spore Apparatus of *Azolla*[1]

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In 1928 Zetsche and Huggler used the term sporopollenin to describe the exine (outer coat material) obtained from either spores or pollen grains (Shaw, 1971). This material, which is comprised of oxidative, cross-linked polymers of carotenoids and/or carotenoid esters (Brooks & Shaw, 1968; Gooday et al., 1974), is resistant to ultraviolet irradiation, desiccation, biological decay, and non-oxidative chemical attack (Shaw, 1971; Strohl et al., 1977). The extensive work of Zetsche and co-workers (1928–1937), and the later studies of Shaw and co-workers (1964–1974), summarized by Brooks and Shaw (1971) and Shaw (1971), have provided substantial information on the chemistry, biochemistry, and geochemistry of sporopollenin.

Sporopollenin has a wide taxonomic distribution, occurring in myxobacteria, fungi, algae, pteridosperms, gymnosperms, and angiosperms (Brooks, 1971; Atkinson et al., 1972; Gooday et al., 1974; Strohl et al., 1977; Honegger & Brunner, 1981). Sporopollenin in sporocarps of *Azolla*—a heterosporous, free-floating fern that occurs worldwide in freshwater habitats (Moore, 1969)—was inferred histochemically (Lucas & Duckett, 1980), but not chemically defined. Therefore, we undertook this effort to verify the presence of sporopollenin in *Azolla* spores, and gain some insight into its contribution to the structure of spore apparatus. We have now identified this compound in *Azolla* spore apparatus by infrared spectroscopy (IR) following acetolysis. The percent (by weight) sporopollenin of the spore apparatus has been determined, and its contribution to spore apparatus architecture demonstrated with scanning electron microscopy prior to and following acetolysis.

Materials and Methods

Azolla mexicana Presl was used as the source of sporocarps. The original population was collected from the Graylodge State Waterfowl Area, Butte County, California, during the summer of 1974 by S. Ela. The culture we utilized was obtained from D. W. Rains and co-workers of the University of California–Davis in 1978, and subsequently maintained at this laboratory. Cultures were grown on nitrogen-free IRRI medium (Peters et al., 1980) and maintained under an illumination of 100–200 $\mu E/m^2/s$, with a 16/8 hr, 26/18°C light-dark regime. Voucher specimens are maintained at the New York Botanical Garden. Mature

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megasporocarps, microsporocarps, and microsporangia were harvested from the frond material by agitation in large volumes of water, and separated from the majority of the frond debris by sieving. Individual spore types were isolated by centrifugation on Percoll discontinuous density gradients at 16,000 × g for 5 min. Microsporangia banded at the 15–30% Percoll interface, microsporocarps banded in the 30% Percoll region, megasporocarps sedimented into the 45% Percoll layer, and the remaining debris formed a pellet on the bottom of the centrifuge tube. The spore types were removed from the gradients and repeatedly washed with water. They were then air dried at room temperature for 48–72 hr, and stored desiccated at room temperature.

The sporopollenin of *Lycopodium* spores has been well characterized by chemical studies (Brooks, 1971; Shaw, 1971) and was employed as a reference material in this study. The *Lycopodium* spores were kindly provided by W. Doyle, University of California–Santa Cruz; the material was originally obtained from Carolina Biological Co. as *Lycopodium* powder, and, as such, may represent a mixture of spores from more than one *Lycopodium* species.

Acetolysis of the dried material was based on the method of Atkinson et al., 1972. The acetolysis residues were ground into a homogenous powder with KBr as a matrix substance, and pelleted at high pressure for use in infrared spectroscopy. Spectra of these samples were recorded on a Beckman IR-20A Infrared Spectrophotometer.

A Perkin-Elmer Model 240 Elemental Analyzer, equipped with an MC-341 Microjector from Control Equipment Corp. for sample automation, was used to analyze dried, pulverized samples of spore material for carbon, hydrogen, and nitrogen.

For scanning electron microscopic (SEM) observations, acetolysis residue material was mounted on aluminum SEM stubs with double-sided tape, and sputter coated with 20 nm platinum. Fresh spore material, which was used for comparison, was fixed in 2% glutaraldehyde in 50 mM PO_4, pH 7.2, dehydrated in a graded acetone series, and critical point dried using liquid CO_2 as the transition fluid. This material was then mounted and coated as indicated above. Examination was performed in an ISI DS-130 scanning electron microscope.

Results and Discussion

Sporopollenin can be defined at the practical level as acetolysis-resistant material (Brooks, 1971; Atkinson, 1972). IR spectra of acetolysis-resistant material obtained from *Azolla* spore apparatus compare very well with those obtained for *Lycopodium* spores carried through the same procedure (Fig. 1), as well as with published spectra for *Lycopodium* sporopollenin (Atkinson et al., 1972; Honegger & Brunner, 1981). The minor differences between the spectra are probably due either to the fact that sporopollenin preparations exhibit variability since their precursors (carotenoids and/or carotenoid esters) vary among organisms (Strohl et al., 1977), or to technical difficulties in grinding the acetolysis-resistant material into a homogenous powder in preparation for IR analysis (Honegger & Brunner, 1981). The band at approximately 2350 cm^{-1} could be due to

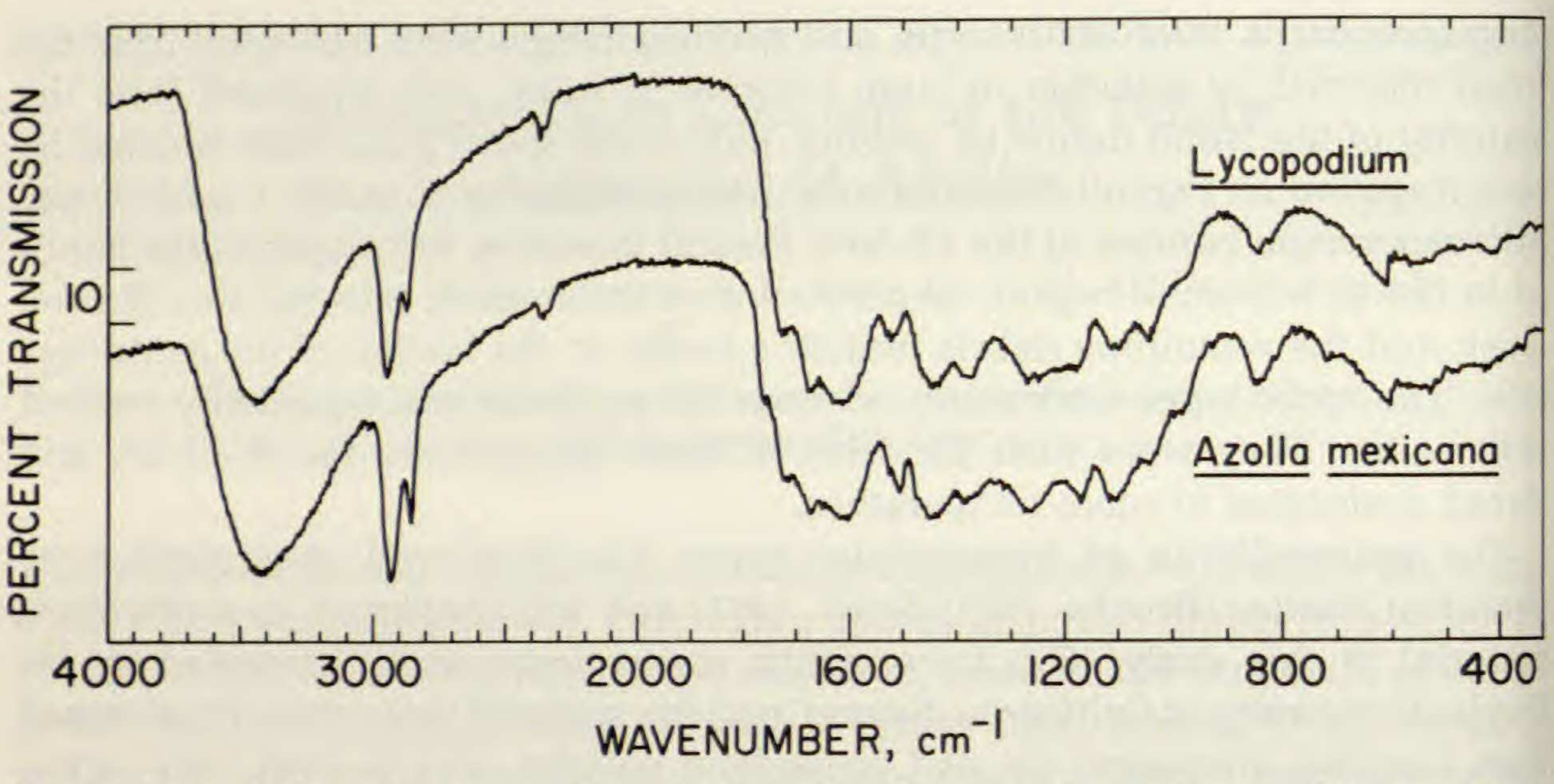


FIG. 1. Infrared absorption spectra of acetolysis residues from *Azolla mexicana* and *Lycopodium*.

the presence of a silicon hydride. This band occurs in spectra reported in the literature, but has not been discussed. An independent laboratory analysis (Galbraith Laboratories, Inc., Knoxville, Tennessee) of our dried *Azolla* sporocarps showed 0.30% silicon on a dry weight basis. Our acetolysis-resistant preparations from both *Azolla* and *Lycopodium* were completely solubilized by 20% chromic acid. This result is consistent with the properties of sporopollenin, as well as with the occurrence of Si as only a very minor component (Atkinson, 1972; Strohl et al., 1977; Good & Chapman, 1978).

Sporopollenin content of *Azolla* megasporocarps, microsporocarps, and microsporangia is higher than that found in *Lycopodium* spores (Table 1). A sporopollenin content of 24% for *Lycopodium* spores agrees exceptionally well with previous reports of 23.4 to 23.8% (Brooks, 1971; Brooks & Shaw, 1971). The highest previously reported value of 31.8% for *Selaginella kraussina* spores (Brooks, 1971) is approximated by the value we obtained for sporopollenin content of *Azolla* microsporangia (30%), while it is exceeded by the values obtained for megasporocarps (43%) and microsporocarps (45%).

The carbon, hydrogen, and nitrogen content of *Azolla* spore apparatus and *Lycopodium* spores was determined before and after acetolysis (Table 2). Following acetolysis the preparations were essentially N-free, consistent with the

TABLE 1. Sporopollenin Content of Various *Azolla* Spore Apparatus (and *Lycopodium* Spores as a Standard).

Sample	% Sporopollenin
A. mexicana megasporocarps	43
A. mexicana microsporocarps	45
A. mexicana microsporangia	30
Lycopodium spores	24

TABLE 2. CHN Content of Various *Azolla* Spore Apparatus (and *Lycopodium* Spores as a Standard) Before (a) and After (b) Acetolysis.

Sample	% C		% H		% N	
	a	*b*	*a*	*b*	*a*	*b*
A. mexicana megasporocarps	59.1 ± 1.0	60.3 ± 1.2	8.4 ± 0.9	5.5 ± 0.0	3.7 ± 0.3	0.1 ± 0.0
A. mexicana microsporangia	58.4 ± 0.7	58.0 ± 0.4	8.2 ± 0.4	5.2 ± 0.0	1.0 ± 0.2	0.0 ± 0.0
Lycopodium spores	67.5 ± 0.4	63.1 ± 0.2	9.9 ± 0.1	5.4 ± 0.0	1.3 ± 0.0	0.0 ± 0.0

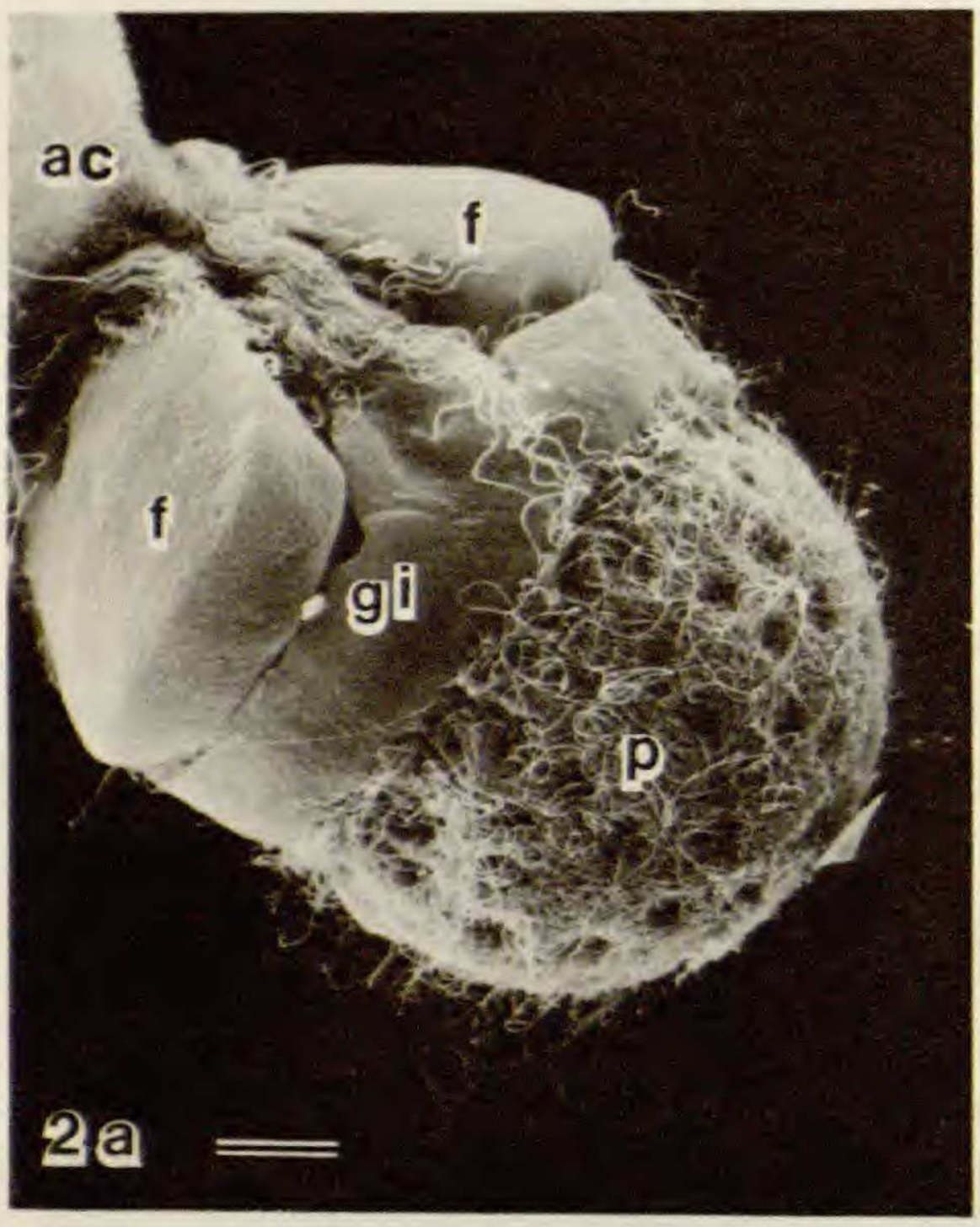
composition of all known sporopollenins (Brooks & Shaw, 1968, 1971; Shaw, 1971). From this analysis we postulate that *Azolla* sporopollenin has an empirical formula of $C_{90}H_{132}O_{33}$ (arbitrarily expressed in terms of C_{90} units, cf. Brooks & Shaw, 1971; Shaw, 1971), assuming that only carbon, hydrogen and oxygen are present. This formula is well within the range of published values for sporopollenins from different sources (Shaw, 1971).

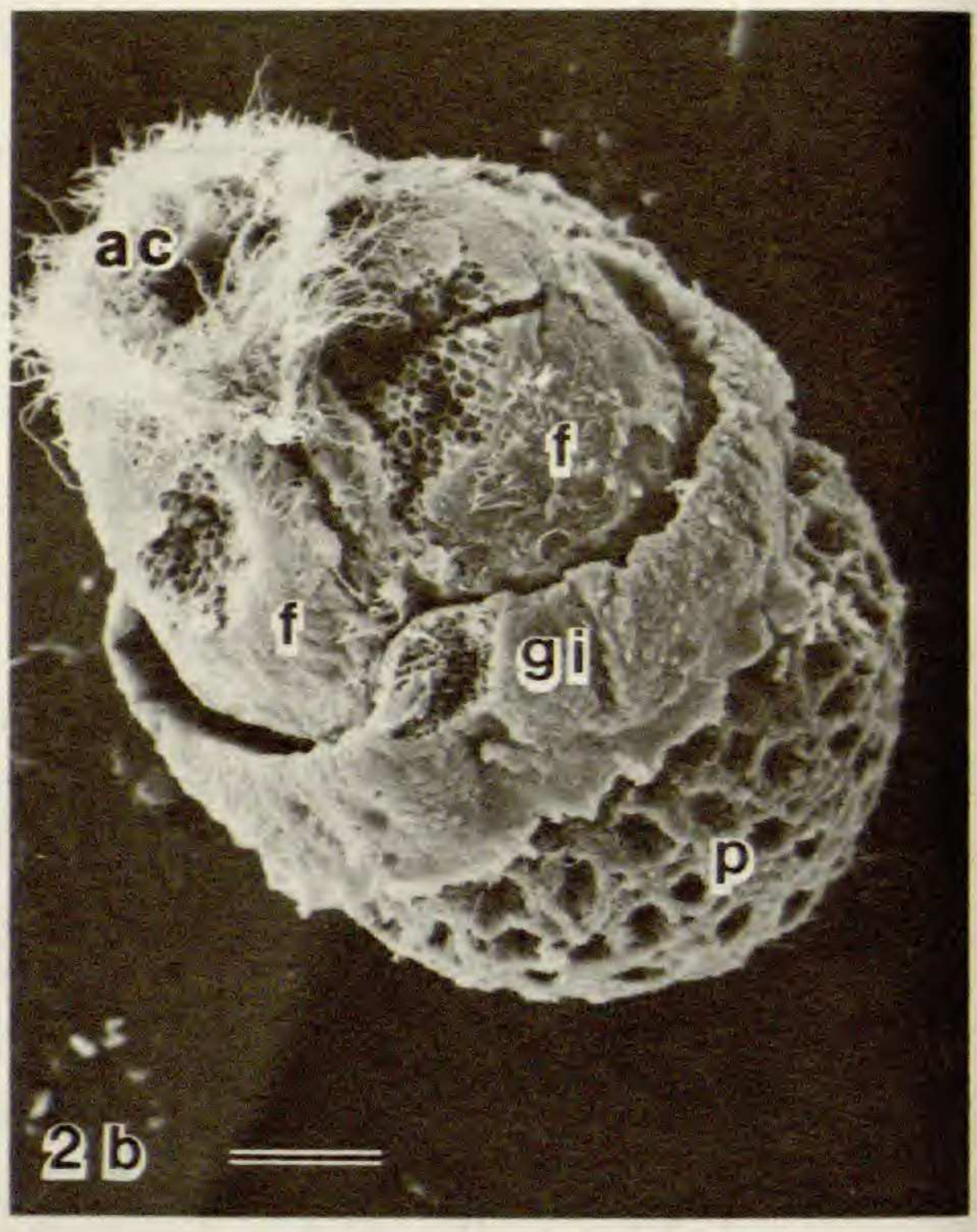
Having conclusively demonstrated that the acetolysis-resistant component of the *Azolla* spore apparatus is sporopollenin, the effect of acetolysis on morphology was assessed with SEM. In general, the overall morphological characteristics are remarkably similar before and after treatment. This implies that the structures have a large sporopollenin content, thus corroborating our chemical analysis.

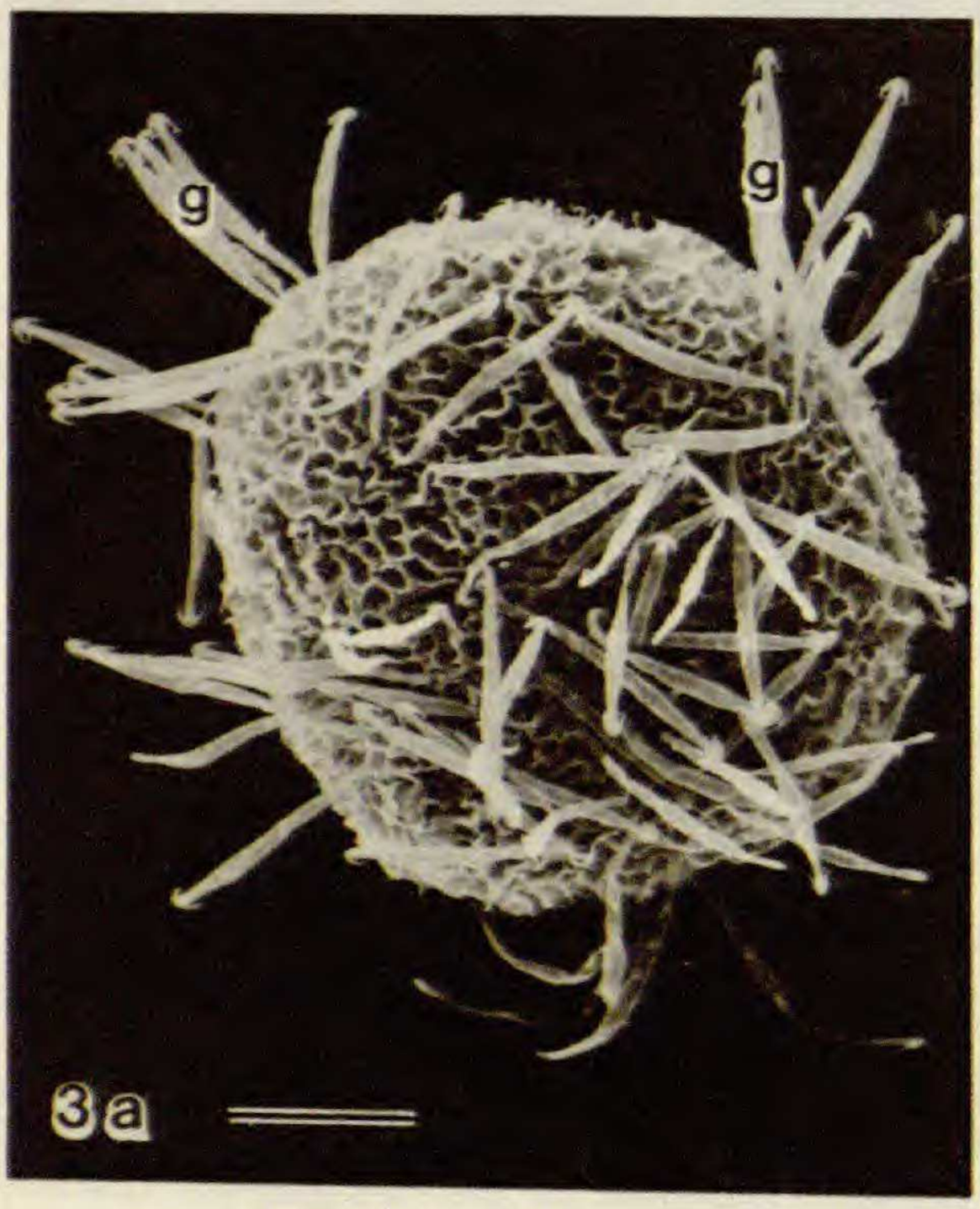
A megasporocarp of *A. mexicana* with the indusium removed (Fig. 2a) reveals four external structures: the perine covering the megaspore, the girdle above the perine, the floats which are recessed into the girdle, and the apical cap sitting atop the floats. The surface of the perine is composed of an entwined labyrinth of filamentous processes with localized areas devoid of these excrescences, giving the perine a dimpled appearance. Long filamentous hairs extend over the surface of the perine and comprise the capture mechanism (Calvert et al., 1983). SEM observations following acetolysis (Fig. 2b) showed the megasporocarps to be somewhat shrunken; however, the gross morphology was unchanged. The fine structure of the perine appeared less distinct, with the filamentous hairs of the capture mechanism fused to the perine surface. The girdle and floats appeared collapsed and contracted, with the floats deeply recessed into the girdle. Dissection of the surface of the floats (Fig. 2b) revealed that the internal alveolate organization was not disrupted by acetolysis.

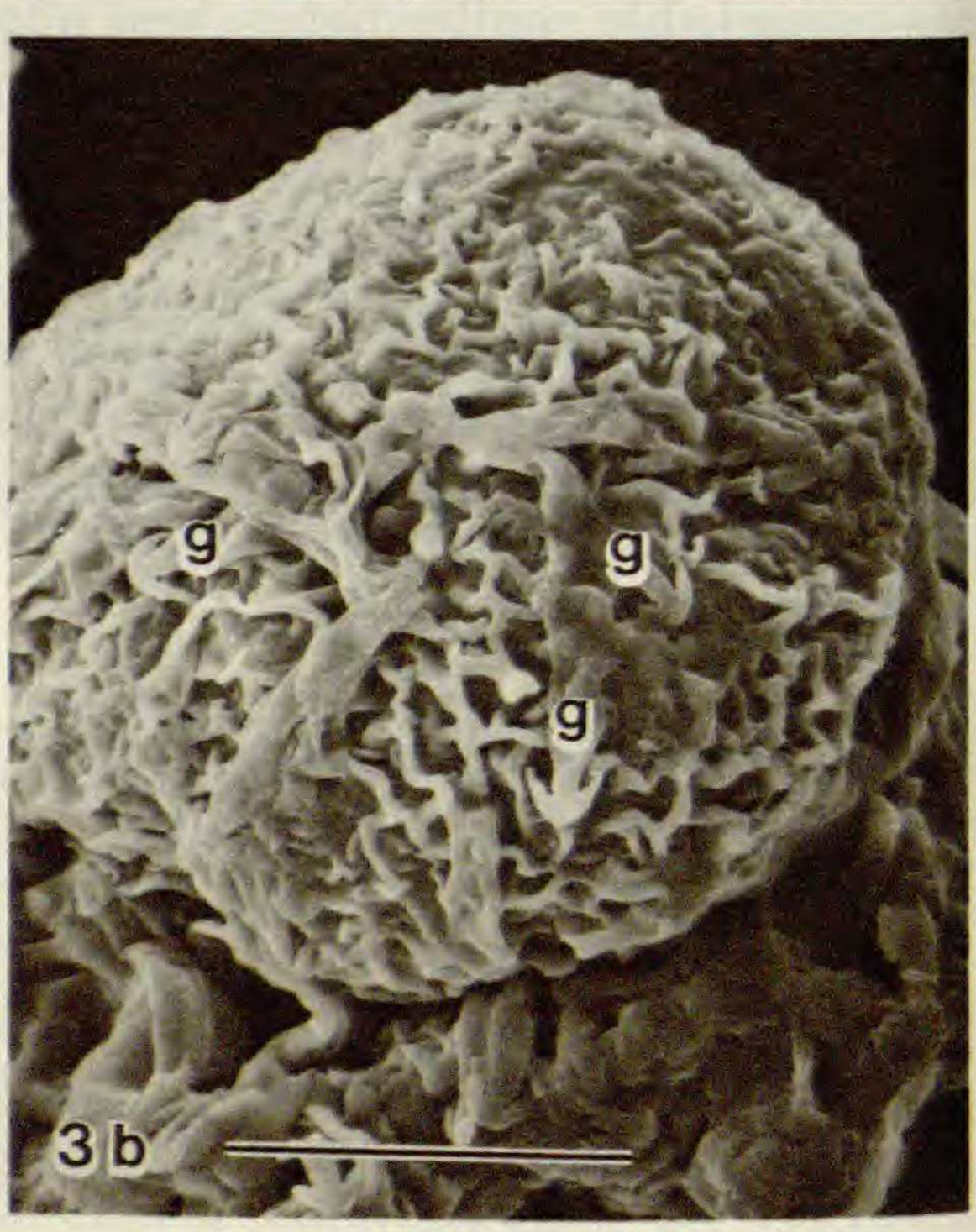
Microsporocarps contain microsporangia which in turn contain massulae. The massulae are pseudocellular, alveolate masses thought to be composed almost entirely of sporopollenin (Lucas & Duckett, 1980). The surface of each massula bears elongate processes termed glochidia. As with the megasporocarp, the gross morphology of the massulae was quite similar before (Fig. 3a) and after (Fig. 3b) acetolysis. However, while Lucas and Duckett (1980) indicated that the massulae and glochidia were strongly resistant to acetolysis, our results suggest a moderate susceptibility. Following acetolysis the alveolate nature of the massular surface was less distinct and, while discernible, the glochidia had collapsed onto the surface of the massula.

These studies do not allow us to state whether any specific structure contains









FIGS. 2 and 3. Scanning electron micrographs of *Azolla mexicana* spore apparatus. Scale bars = 50 μm. 2, Megasporocarp before (a) and after (b) acetolysis, showing the perine (p), girdle (gi), floats (f), and apical cap (ac). 3, Intact massula from microsporocarp, before (a) and after (b) acetolysis, showing glochidia (g).

only sporopollenin, or for that matter, no sporopollenin. However, they have documented the presence of sporopollenin by IR spectroscopy, and have clearly shown that it is a major structural component of *Azolla* sporocarps, with levels of sporopollenin significantly exceeding those reported previously.

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